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published in
Journal of Biological Chemistry
2005

DOI (link to publisher)
10.1074/jbc.M504165200

document version
Publisher's PDF, also known as Version of record

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 41, pp. 34741–34746, October 14, 2005

A $G_{q/11}$-coupled Mutant Histamine H_1 Receptor F435A Activated Solely by Synthetic Ligands (RASSL)*

Received for publication, April 18, 2005, and in revised form, July 5, 2005 Published, JBC Papers in Press, July 18, 2005, DOI 10.1074/jbc.M504165200

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Recently, G protein-coupled receptors activated solely by synthetic ligands (RASSLs) have been introduced as new tools to study $G\alpha_i$ signaling *in vivo* (1, 2). Also, $G\alpha_s$-coupled G protein-coupled receptors have been engineered to generate $G\alpha_s$-coupled RASSLs (3, 4). In this study, we exploited the differences in binding pockets between different classes of H_1 receptor agonists and identified the first $G\alpha_{q/11}$-coupled RASSL. The mutant human H_1 receptor F435A (6.55) combines a strongly decreased affinity (25-fold) and potency for the endogenous ligand histamine (200-fold) with improved affinities (54-fold) and potencies (2600-fold) for 2-phenylhistamines, a synthetic class of H_1 receptor agonists. Molecular dynamics simulations provided a mechanism for distinct agonist binding to both wild-type and F435A mutant H_1 receptors.

Receptors activated solely by synthetic ligands (RASSLs)[2] have recently been developed as tools to control G protein signaling *in vivo* (5). RASSLs are G protein-coupled receptors (GPCRs) that no longer respond to their endogenous ligands but can still be activated by synthetic ligands (5). Given the biological importance of GPCRs, the ability to stimulate a single G protein pathway in a tissue of choice *in vivo* is useful for the understanding of subsequent changes in downstream signaling. The use of RASSLs has already provided valuable insight into the effect of $G\alpha_i$ signaling in the heart. Stimulation of a $G\alpha_i$-coupled RASSL selectively expressed in the murine heart resulted in a strong (3-fold) and rapid (within 1 min after drug administration) decrease in heart rate (1). In the future, RASSLs may further be applied, *e.g.* to create reversible models of disease states, or combined with microarrays, to result in gene expression fingerprints of specific G protein pathways (for review, see Ref. 6).

There are four major classes of G proteins, named after their respective α-subunit ($G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$, and $G\alpha_{12}$). Each class couples to specific signal transduction pathways, which are well characterized *in vitro* (for a review, see Ref. 7). The first described RASSL couples through $G\alpha_i$ proteins (5) and thus far is the only RASSL being applied *in vivo* (1, 2, 8). Also, more recently, several $G\alpha_s$-coupled RASSLs have been developed (3, 4). Thus far, there have been no reports about either $G\alpha_{q/11}$- or $G\alpha_{12}$-coupled RASSLs.

The human histamine H_1 receptor (H_1R) couples through $G\alpha_{q/11}$ proteins and thereby activates phospholipase C, resulting in, for example, inositol phosphate hydrolysis and increased concentrations of intracellular Ca^{2+} (9, 10). We previously demonstrated that the H_1 receptor also activates NF-κB, both in a ligand-induced as well as in a constitutive manner, via $G\alpha_{q/11}$ proteins (11). Although several $G\alpha_q$-coupled GPCRs also activate $G\alpha_{12}$ (for a review, see Ref. 12), the H_1R does not seem to couple to $G\alpha_{12}$ (10, 11, 13).

By mutational analysis, we previously characterized several amino acids that are involved in the binding of histamine and several synthetic agonists, such as the histaprodifens (a new class of specific H_1R agonists) (14). Mutation of Phe^{435} (6.55) to Ala in TM6 (transmembrane domain 6) resulted in a strong decrease in affinity and potency of histamine, whereas only marginally affecting affinities and potencies of histaprodifens. In this study, we showed that for another class of H_1R agonists, the substituted 2-phenyl-histamines (PheHAs, Ref. 15), mutation of F435A results in strongly increased affinities and potencies, thereby identifying the first $G\alpha_{q/11}$-coupled RASSL.

EXPERIMENTAL PROCEDURES

Materials—pNF-κB-Luc was obtained from Stratagene (La Jolla, CA). ATP disodium salt, bovine serum albumin, chloroquine diphosphate, DEAE-dextran (chloride form), histamine dihydrochloride, mepyramine (pyrilamine maleate), glycerol, Triton X-100, and polyethyleneimine were purchased from Sigma. D-Luciferin was obtained from Duchefa Biochemie BV (Haarlem, The Netherlands). Cell culture media, penicillin, and streptomycin were obtained from Invitrogen. Fetal bovine serum was obtained from Integro B. V. (Dieren, The Netherlands). Cell culture plastics were obtained from Corning Costar (Corning, NY). [^{3}H]Mepyramine (20 Ci/mmol) was purchased from ICN Biomedicals B. V. (Zoetermeer, The Netherlands). 2-(3-Chlorophenyl)histamine, 2-(3,4-dichlorophenyl)histamine, 2-(cyclohexyl)-histamine, 2-(methylcyclohexyl)-histamine, and 2-(ethylcyclohexyl)-histamine were synthesized at the Vrije Universiteit Amsterdam, The Netherlands. Gifts of 2-(3-trifluoromethyl)phenylhistamine dihydrogenmaleate (Dr. W. Schunack), pcDEF3 (Dr. J. Langer, Ref. 16), and the cDNA encoding the human H_1 receptor (Dr. H. Fukui, Ref. 17) are greatly acknowledged.

Cell Culture and Transfection—COS-7 African green monkey kidney cells were maintained at 37 °C in a humidified 5% CO_2, 95% air atmosphere in Dulbecco's modified Eagle's medium containing 50 IU/ml penicillin, 50 μg/ml streptomycin, and 5% (v/v) fetal bovine serum. COS-7 cells were transiently transfected using the DEAE-dextran method as described previously (11).

Site-directed Mutagenesis—The cDNA encoding the human H_1R (17) was subcloned in the pAlter plasmid (Promega), and point mutations were created according to the manufacturer's protocol (Altered Sites® II, Promega). cDNA of all mutant and wild-type receptors were subcloned into the expression plasmid pcDEF3 (16). Mutations in the

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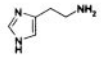
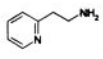
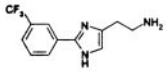
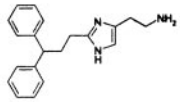
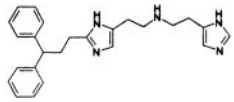
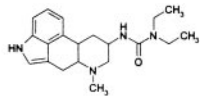
[2] The abbreviations used are: RASSL, receptor activated solely by synthetic ligands; H_1R, histamine H_1 receptor; WT, wild-type; HA, histamine; PheHA, 2-phenylhistamine; ClPheHA, 2-(3-chlorophenyl)histamine; Cl_2PheHA, 2-(3,4-dichlorophenyl)histamine; dClPheHA, 2-(3,4-dichlorophenyl)histamine; CF_3PheHA, 2-(3-trifluoromethyl-phenyl-)histamine; CxHA, 2-cyclohexyl-histamine; MeCxHA, 2-(methylcyclohexyl)-histamine; EthCxHA, 2-(ethylcyclohexyl)-histamine; COS-7, African green monkey kidney cells; GPCR, G protein-coupled receptors; MD, molecular dynamics.

TABLE ONE

Affinities of H_1R agonists for wild-type and Phe^{435} Ala mutant H_1Rs

	Agonist affinity (pK_i)[a]					
	Histamine	Pyridylethylamine	2-(3-trifluoromethyl-phenyl)histamine	Histaprodifen	Suprahistaprodifen	8R-Lisuride
						
WT	4.1 ± 0.1	3.8 ± 0.1	5.0 ± 0.1	5.6 ± 0.1	5.8 ± 0.1	7.1 ± 0.1
Phe^{435}Ala	2.7 ± 0.1[c]	3.6 ± 0.1	6.8 ± 0.1[c]	5.3 ± 0.1[b]	5.5 ± 0.1[b]	6.7 ± 0.1

[a] Affinities are determined by [^{3}H]mepyramine displacement. All data are calculated as the mean ± S.E. of at least three experiments, each performed in triplicate.
[b] $p < 0.01$.
[c] $p < 0.001$ *vs.* WT H_1R.

cDNA were verified by DNA sequencing using the dideoxy chain termination method.

NF-κB Reporter Gene Assay—Cells transiently co-transfected with pNF-κB-Luc (125 μg/1 · 10^7 cells) and pcDEF3 containing mutant or wild-type human H_1R cDNA (25 μg/1 · 10^7 cells) were seeded in 96-well white plates (Costar) in serum-free culture medium and incubated with drugs. After 48 h, cells were assayed for luminescence by aspiration of the medium and the addition of 25 μl/well luciferase assay reagent (0.83 mM ATP, 0.83 mM D-luciferin, 18.7 mM $MgCl_2$, 0.78 μM $Na_2H_2P_2O_7$, 38.9 mM Tris (pH 7.8), 0.39% (v/v) glycerol, 0.03% (v/v) Triton X-100, and 2.6 μM dithiothreitol). After 30 min, luminescence was measured for 3 s/well in a Victor^2 (Wallac).

Histamine H_1R Binding Studies—The transfected COS-7 cells used for radioligand binding studies were harvested after 48 h and homogenized in ice-cold 50 mM Na_2/K phosphate buffer (pH = 7.4) (binding buffer). The COS-7 cell homogenates were incubated for 30 min at 30 °C in H_1R binding buffer in 200 μl with 3 nM [^{3}H]mepyramine. The nonspecific binding was determined in the presence of 1 μM mianserin. The incubations were stopped by rapid dilution with 3 ml of ice-cold H_1R binding buffer. The bound radioactivity was separated by filtration through Whatman GF/C filters that had been treated with 0.3% polyethylenimine. Filters were washed twice with 3 ml of H_1R binding buffer, and radioactivity retained on the filters was measured by liquid scintillation counting. Binding data were evaluated by a non-linear, least squares curve-fitting procedure using GraphPad Prism® (GraphPad Software, Inc., San Diego, CA).

Analytical Methods—Protein concentrations were determined according to Bradford (18), using bovine serum albumin as a standard. All data shown are expressed as means ± S.E. Statistical analyses were carried out by non-paired Student's *t* test. *p* values < 0.05 were considered to indicate a significant difference (*a*, $p < 0.05$; *b*, $p < 0.01$; *c*, $p < 0.001$).

Molecular Modeling—Our H_1R homology model was obtained using the bovine rhodopsin crystal structure (Protein Data Bank entry 1L9H, Ref. 19) as the template. The third intracellular loop that connects transmembranes 5 and 6 was omitted due to its large size. Side chains were added using the homology module of InsightII (20). Water molecules present in the crystal structure were not incorporated. The initial model was refined by a steepest descent energy minimization. The minimized model was placed in a dodecahedral box filled with simple point charge water (21), and a second minimization step using steepest descent was performed. Hereafter, a 20-ps molecular dynamics (MD) run was performed with positions restraints (1000 kJ · mol^{-1} nm^{-2}) on all heavy protein atoms. Finally, the model was refined by a 100-ps MD run. In this structure, Phe^{435} was changed to Ala with InsightII, thus creating a model of the F435A H_1R.

Histamine and 2-(3-chlorophenyl)histamine (ClPheHA) were docked in the WT and F435A models using AutoDock 3.0.0 (22) applying default parameters. The monocationic ligands were assigned Löwdin atomic charges obtained after a single point *ab initio* restricted Hartree-Fock calculation using 6-31Gr* with the GAMESS US package (23). The protein was assigned with KOLLUA partial charges using the SYBYL program (version 6, Tripos, St. Louis). Only essential hydrogens were taken into account.

The obtained ligand-receptor complexes were minimized using the steepest descent method. These ligand-receptor complexes were placed in a dodecahedral box, with a minimum distance of 7 Å between protein and the box. The system was solvated with simple point charge water, and the protein partial charges were assigned by GROMACS.

Again, a steepest descent procedure was performed on the system. Subsequently, a set of MD runs with position restraints (1000 kJ· $mol^{-1}nm^{-2}$) was applied to the system in which a controlled release of the restraints was performed. A run for 50 ps with position restraints on all heavy protein atoms and all ligand atoms was performed, with the protein hydrogens and all water molecules unrestrained. Consecutively, a run was performed for 20 ps with position restraints on all Cα-atoms, all heavy atoms of residues Asp^{107} and Asn^{198}, and all ligand atoms. Finally, a MD run was performed for 1 ns with only position restraints on Cα-atoms. During the last 500 ps of this run, the presence of hydrogen bonds was analyzed using cutoff distance between heavy atoms of 3.5 Å and a cutoff angle between acceptor donor and hydrogen of 60°.

All minimizations and MD simulations were performed using the GROMACS software package and the GROMOS 43a1 force field (24, 25) and LINCS constraints (26) on all bonds. An NPT ensemble was generated using both the Berendsen thermostat and the barostat with default settings for 300 K and 1 bar (25).

RESULTS

Mutation F435A Differentially Modulates Affinities of Several Classes of H_1R Agonists—At the H_1R, several H_1R agonists show a higher affinity than the natural agonist histamine. The histamine analogs 2-(3-trifluoromethyl-phenyl)histamine (CF_3PheHA), histaprodifen, suprahistaprodifen, and the newly characterized H_1R agonist 8R-lisuride bind 9–1000-fold better to the H_1R as compared with histamine (TABLE ONE). Previously, we characterized Phe^{435} (6.55), as a specific interaction point for histamine (14). Mutation of this residue into alanine resulted in a 25-fold decrease in histamine affinity without drastic alterations in the affinity of the agonists histaprodifen and suprahistapodifen

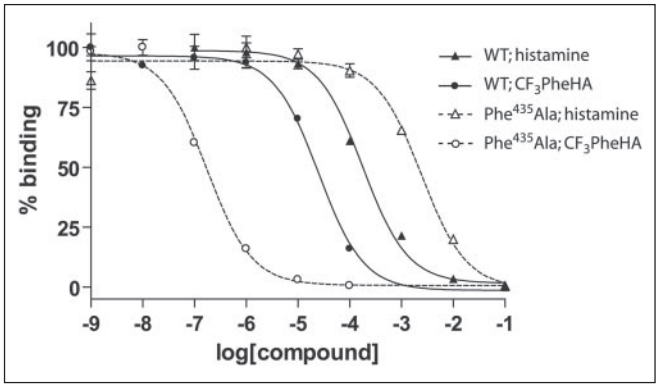


FIGURE 1. **Displacement of [^{3}H]mepyramine binding to wild-type H_1R (*closed symbols, solid lines*) and to mutant receptor H_1R F435A (*open symbols, dotted lines*) receptors by histamine (*triangles*), and 2-(3-trifluoromethyl-phenyl)histamine (CF_3PheHA, *circles*).** A representative experiment is shown.

(TABLE ONE) (Ref. 14). In this study, we further studied the role of Phe^{435} (6.55) in agonist binding to the H_1R. TABLE ONE shows that, similar to histaprodifen and suprahistaprodifen, affinities of pyridylethylamine as well as 8R-lisuride are comparable for WT and F435A mutant H_1Rs. Unexpectedly, for F435A H_1R, the affinity of CF_3PheHA was increased 54-fold as compared with WT H_1R (Fig. 1 and TABLE ONE). Thus, the F435A mutant H_1R has an affinity for CF_3PheHA that exceeds the affinity of histamine more than 10,000-fold.

Basic Characterization of Phe^{435} Mutant H_1Rs—Phenylalanine at position 6.55 is conserved among all known H_1Rs (27) and is implicated in ligand binding in both human and guinea pig H_1Rs (14, 28). The F435A mutation removes an aromatic ring structure from the binding pocket, and therefore, potential interactions with either ligands or other amino acids in the H_1R-binding pocket may be lost. However, exchanging the bulky Phe^{435} (6.52) with the smaller alanine may also increase the size of the ligand-binding pocket and thus create space for the trifluoromethyl-phenyl moiety of CF_3PheHA, resulting in an increased affinity for this ligand. To address the latter possibility, we created additional mutant H_1Rs (F435V and F435L) that vary in the size of the side chain at position 6.55.

The generated mutant receptors are expressed at comparable levels (B_{max} = 13.6–20.1 pmol/mg of protein) in transiently transfected COS-7 cells and bind the H_1R radioligand [^{3}H]mepyramine with virtually unchanged affinity (K_d = 1.5–5.7 nM), as determined by radioligand saturation binding experiments (TABLE TWO). Although the expression levels are similar, there is a clear difference in constitutive GPCR activities of the tested H_1R mutants as measured by an NF-κB-driven reporter gene assay (Fig. 2, *white bars*). Although the basal activity of H_1R F435L H_1R is comparable with the WT H_1R, the level of constitutive NF-κB activation of H_1R F435V is twice as high, whereas for mutant H_1R, F435A constitutive activity is hardly detectable. Although the levels of constitutive NF-κB activation vary between the mutant H_1Rs, their agonist-induced responses are comparable (Fig. 2, *black bars*), indicating that G protein-coupling of the mutant H_1Rs is not impaired.

Binding Analysis of WT and Phe^{435} Mutant H_1Rs—Similar to the mutant F435A H_1R (pK_i = 2.7), the mutant H_1R F435V (pK_i = 2.9) and F435L (pK_i = 2.9) exhibit a strong decrease in their affinity for histamine as compared with the WT receptor (pK_i = 4.1) (TABLE THREE). Again, these mutant receptors exhibit an increased affinity for CF_3PheHA. The exchange of Phe^{435} for leucine, valine, or alanine results in mutant H_1Rs with a 47-, 124-, and 54-fold increased affinity for CF_3PheHA respectively, as compared with the WT receptor (pK_i = 5.0) (TABLE THREE). For these mutant H_1Rs, the affinity of CF_3PheHA greatly exceeds their affinity of the endogenous ligand histamine, the mutant F435V H_1R exhibiting the greatest selectivity for the synthetic agonist CF_3PheHA over histamine (17,000-fold).

We tested two other substituted PheHAs, ClPheHA and 2-(3,4-dichlorophenyl)histamine (Cl_2PheHA), on the mutant H_1Rs as well. For the WT H_1R, the affinity of ClPheHA (pK_i = 5.3) is slightly higher then than that of CF_3PheHA (pK_i = 5.0). An (additional) *para*-chloro substituent is not favorable for H_1R affinity (pK_i = 4.6), fitting previous findings for both human and rodent H_1Rs (29). Similar to CF_3PheHA, the affinity of ClPheHA is increased upon mutation of Phe^{435} into leucine (pK_i = 6.8), valine (pK_i = 7.3), or alanine (pK_i = 6.7). Although for Cl_2PheHA, H_1R affinities are lower and increases in affinity are smaller, the same trend upon mutation is observed (pK_i values, 4.6, 5.3, 5.8, and 5.4, respectively). Similar to CF_3PheHA, for ClPheHA and Cl_2PheHA, mutation F435V also results in the largest increase in affinity, as compared with the WT receptor.

Additionally, we tested a series of 2-cyclohexyl-histamines (CxHAs), varying in spacer length between the imidazole ring and the cyclohexyl moieties: CxHA, 2-(methyl-cyclohexyl)-histamine (MeCxHA), and 2-(ethyl-cyclohexyl)-histamine (EthCxHA). The affinities of the CxHAs are approximately equal to the affinity of histamine (TABLE THREE). Again, mutation of Phe^{435} into alanine, valine, or leucine results in increased affinities as compared with the WT H_1R (TABLE THREE). The increases in affinity, however, are smaller (maximum 10-fold) than those observed for the PheHAs (maximum 120-fold). Although changes in affinity are less prominent, for EthCxHA, we observe a correlation between the space available in the binding pocket (Ala → Val → Leu → Phe) and the affinities for the mutant receptors (F435A, pK_i 4.9; F435V, pK_i = 4.6; F435L, pK_i = 4.3; and WT, pK_i = 4.1, TABLE THREE). For both CxHA and MeCxHA, the highest affinities are observed for the F435V mutant H_1R, following the trend observed for the PheHAs.

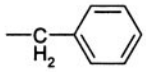
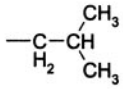
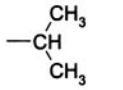
Functional Analysis of WT and Phe^{435} Mutant H_1Rs—Using an NF-κB-driven reporter gene assay, we evaluated the potencies of several 2-phenylsubstituted histamine analogs for mutant and WT H_1Rs. For the WT H_1R, these analogs are less potent than the endogenous ligand histamine (pEC_{50} = 6.9) (TABLE THREE). Fig. 3 clearly illustrates that as compared with the WT H_1R, the potency of HA at the H_1R F435A is decreased 200-fold, whereas the potency of ClPheHA is increased 2,600-fold. Also, for CF_3PheHA and Cl_2PheHA, potencies were increased upon the mutation F435A; again ClPheHA (pEC_{50} = 9.4) and CF_3PheHA (pEC_{50} = 9.2) are equipotent, and Cl_2PheHA is clearly less potent (pEC_{50} = 7.9) (TABLE THREE). For mutant H_1Rs F435L and F435V, similar trends are observed, with PheHA potencies for H_1R F435V being slightly higher than for F435A and F435L H_1Rs. For mutant H_1Rs, F435A, F435V, and F435L ClPheHA and CF_3PheHA are agonists with subnanomolar potencies, which exceed the potency of histamine 70,000–280,000-fold.

Molecular Modeling of WT and F435A Mutant H_1Rs—Our model of the H_1R, based on the bovine rhodopsin crystal structure, was used to visualize the interactions of either histamine or ClPheHA with the WT and F435A mutant H_1R. Models of both WT and F435A H_1Rs were optimized by molecular dynamics simulations. Hereafter, histamine and ClPheHA were automatically docked in both WT and F435A mutant H_1R models. Finally, a second round of molecular dynamics was used to optimize the receptor-ligand complexes. In the WT H_1R, both histamine and ClPheHA show an ionic interaction between the conserved Asp^{107} (3.32) in TM3 (transmembrane domain 3) and their ethylamine group over distances of 2.6 and 3.0 Å, respectively (Fig. 4, *A* and *C*). As expected, differences between histamine and ClPheHA can be observed for interaction with Phe^{435}. In binding histamine, Phe^{435} has

TABLE TWO

Characteristics of [^{3}H]mepyramine binding to WT and mutant H_1R

The values are determined by saturation radioligand binding assays. All data are calculated as the mean ± S.E. of at least three experiments, each performed in triplicate.

	Amino acid	K_d [^{3}H]mepyramine (nM)	H_1 receptor density (pmol/mg protein)
WT		1.6 ± 0.1	20.1 ± 6.7
Phe^{435}Leu		1.5 ± 0.1	15.0 ± 3.7
Phe^{435}Val		1.7 ± 0.1	22.0 ± 7.5
Phe^{435}Ala		5.7 ± 1.0[a]	13.6 ± 6.4

[a] $p < 0.01$ *vs.* WT receptor.

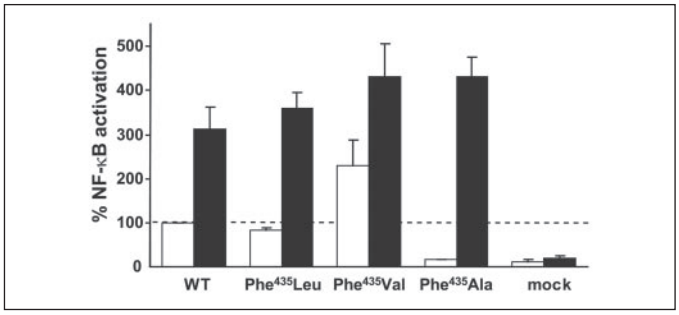


FIGURE 2. **Basal H_1R activation (*white bars*) and maximal histamine-induced NF-κB activation (*black bars*) in COS-7 cells transfected with cDNA of WT, mutant H_1Rs, or empty vector (*mock*).** Basal activity of WT H_1R is put at 100%. Values are determined by NF-κB-driven reporter gene assays.

an indirect role, keeping Phe^{432} in an edge-to-face orientation toward the imidazole moiety of histamine (Fig. 4*A*). In this orientation, a hydrogen bond can be formed between the distal nitrogen of the imidazole ring of histamine and Asn^{198} at a distance of 2.7 Å and is observed during 47% of the MD simulation. In binding ClPheHA, Phe^{435} directly interacts with the ligand by $\pi-\pi$ stacking with the phenyl moiety of ClPheHA (Fig. 4*B*). By this interaction, Phe^{435} prevents hydrogen bonding of the imidazole ring with Asn^{198} since the distance exceeds 5 Å.

The difference in interaction with Phe^{435} between histamine and ClPheHA is further illustrated by observed changes in receptor-ligand interactions upon the mutation F435A. For histamine, the mutation F435A releases Phe^{432}, thereby allowing histamine to shift toward Asp^{107}, resulting in an increased distance between the agonist and Asn^{198} (7.0 Å) and the loss of the hydrogen bond between Asn^{198} and the imidazole ring of histamine (Fig. 4*C*). Upon the mutation F435A, we observed an opposite effect for ClPheHA; removal of the $\pi-\pi$ stacking with Phe^{435} allows the ligand to move deeper in the binding pocket, enabling hydrogen bonding of the imidazole ring with Asn^{198} at a distance of 2. 4 Å. This hydrogen bond is observed during 78% of the MD simulation (Fig. 4*D*).

DISCUSSION

Most H_1R agonists are structurally closely related to histamine and have the imidazole ring either replaced by other heterocycles (pyridylethylamine, thiozolylethylamine) or substituted at the 2-position (histaprodifens, PheHAs) (30). Already in 1994, we demonstrated that H_1R agonists, although structurally similar, appear to have different binding modes (31). Recently, we extended this conclusion to histaprodifens. In contrast to histamine, for this class of compounds, interactions with either Asn^{198} or Phe^{435} are no longer required for high affinity H_1R binding (14). Asn^{198}, however, still appeared crucial for receptor activation. To establish whether the interaction with Phe^{435} is specific to histamine alone, we tested several other H_1R agonists for interaction with Phe^{435}. Comparable with histaprodifen, the affinities of suprahistaprodifen, pyridylethlamine, and 8R-lisuride were not altered by the mutation F435A. For CF_3PheHA however, the affinity for H_1R F435A was 54-fold higher than for the WT H_1R. Studying receptor-mediated NF-κB activation, the differences in potency of CF_3PheHA between WT and F435A mutant receptors (1,500-fold) even exceeded the observed differences in affinity. Similar trends are observed for the 3-chloro-substituted PheHA (ClPheHA) and to a lesser extent for 3,4-dichloro-substituted PheHA (dClPheHA). These observations strongly suggested that despite structural (histamine-like moiety) and/or functional similarity (agonism), the precise orientation of H_1R agonists within the H_1R-binding pocket differs.

We used computer modeling to examine the different H_1R binding modes for histamine and the synthetic agonist ClPheHA. For histamine, we observed hydrogen bonding between one of the imidazole nitrogens and Asn^{198}, whereas for ClPheHA, this interaction is not observed in our molecular dynamics simulations. We previously demonstrated that interaction with Asn^{198} appears to be a prerequisite for H_1R activation (14). This explains why although the affinity of histamine for the WT H_1R is lower than that of ClPheHA, histamine is more efficacious as an H_1R agonist.

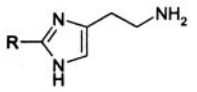
Our computational studies suggested that upon mutation of Phe^{435} to alanine, the aromatic interaction of Phe^{432} with the imidazole ring of histamine is lost. Histamine gained in conformational freedom (entropy) and the distance between Asn^{198}, and the imidazole nitrogen of histamine increased to 7 Å, suggesting the loss of hydrogen bonding. The loss of this interaction and the increase in entropy most likely accounted for the observed decreased in affinity and potency of histamine (as compared with the WT H_1R). In contrast, for ClPheHA, the distance between its imidazole moiety and Asn^{198} was decreased in the F435A H_1R, enabling hydrogen bonding and thus facilitating both high affinity binding and potent activation of the F435A H_1R.

To delineate the effect of mutating the bulky, aromatic Phe^{435} to a

TABLE THREE

Characterization of H_1R agonists on wild-type and mutant H_1Rs

Potencies of agonists (pEC_{50}) are determined by an NF-κB-driven reporter gene assay. Affinities are determined by [^{3}H]mepyramine displacement. All data are calculated as the mean ± S.E. of at least three experiments, each performed in triplicate. ND indicates that the value could not be determined.



R	WT		Phe^{435}Leu		Phe^{435}Val		Phe^{435}Ala	
	pK_i	pEC_{50}	pK_i	pEC_{50}	pK_i	pEC_{50}	pK_i	pEC_{50}
histamine	4.1 ± 0.1	6.9 ± 0.1	2.9 ± 0.1[c]	4.5 ± 0.1[c]	2.9 ± 0.1[c]	4.4 ± 0.2[c]	2.7 ± 0.1[c]	4.6 ± 0.2[c]
2-(3-trifluoromethyl-phenyl)histamine	5.0 ± 0.1	6.0 ± 0.1	6.7 ± 0.1[c]	9.2 ± 0.1[c]	7.1 ± 0.1[c]	9.6 ± 0.1[c]	6.8 ± 0.1[c]	9.2 ± 0.1[c]
2-(3-chlorophenyl)histamine	5.3 ± 0.1	6.2 ± 0.1	6.8 ± 0.1[c]	9.5 ± 0.2[c]	7.3 ± 0.1[c]	9.8 ± 0.1[c]	6.7 ± 0.1[c]	9.4 ± 0.1[c]
2-(3,4-dichlorophenyl)histamine	4.6 ± 0.1	5.0 ± 0.1	5.3 ± 0.1[c]	7.3 ± 0.2[c]	5.8 ± 0.1[c]	8.3 ± 0.1[c]	5.4 ± 0.1[c]	7.9 ± 0.1[c]
2-(cyclohexyl)-histamine	3.8 ± 0.1	ND	4.2 ± 0.1[c]	ND	4.9 ± 0.1c	ND	4.6 ± 0.1[c]	ND
2-(methylcyclohexyl)-histamine	3.9 ± 0.1	ND	4.3 ± 0.1[c]	ND	4.9 ± 0.1[c]	ND	4.7 ± 0.1[c]	ND
2-(ethylcyclohexyl)-histamine	4.1 ± 0.1	ND	4.3 ± 0.1[a]	ND	4.6 ± 0.1[b]	ND	4.9 ± 0.1[c]	ND

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$ *vs.* WT receptor.

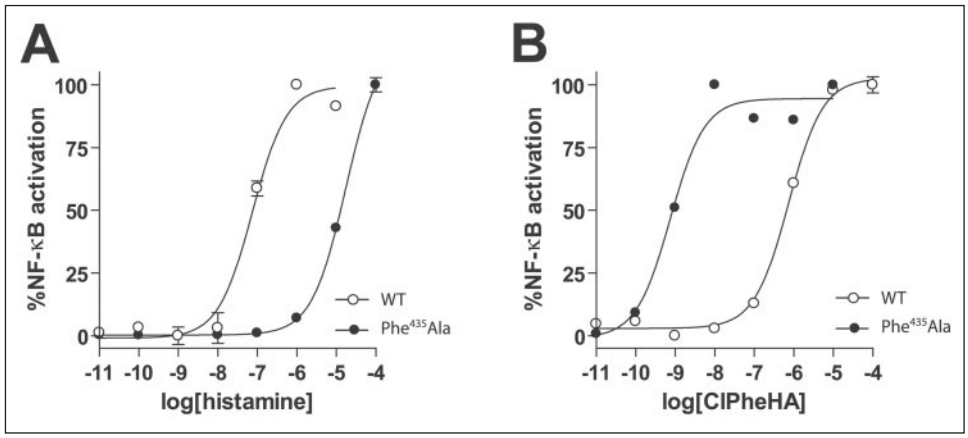


FIGURE 3. **Representative dose response curves of histamine (*A*) and ClPheHA (*B*) for wild-type H_1R (○) and mutant receptor H_1R F435A (●) as measured by NF-κB activation.**

small, aliphatic alanine, mutant H_1Rs were created in which the side chain at this position have intermediate sizes: Phe^{435} → Leu → Val → Ala. For none of the PheHAs did we observe gradual size-dependent changes in affinity or potency. Instead, increased affinities and potencies appeared an all-or-nothing phenomenon, correlating with the presence or absence of the aromatic Phe^{435}. The various mutations also affected affinities of MeCxHA and CxHA, in a similar but attenuated fashion. Since we did observe a small but clear correlation between affinity and side chain bulk at position 6.55 for EthCxHA, having an ethyl chain as a spacer between histamine and cyclohexyl moieties, we speculated that only for this ligand, the space in binding pocket is limiting over the full array of receptors. Potentially, increasing the spacer length between histamine moiety and (substituted) phenyl ring would also result in PheHAs that can discriminate between the various mutant H_1Rs.

RASSLs are GPCRs that no longer respond to endogenous ligands but can still be activated by synthetic ligands (5). Previously, such mutant GPCRs have been introduced as new tools to selectively study specific G protein pathways *in vivo* (1). So far, RASSLs for both G_i and G_s pathways have been described (3–5). For mutant receptors F435A, F435V, and F435L, the affinity and potency of histamine are strongly decreased as compared with the WT H_1R, whereas the affinities and potencies of PheHAs are strongly increased (TABLE THREE). These mutant receptors can therefore be classified as the first $G\alpha_{q/11}$-coupled RASSLs. It is noteworthy that these mutant H_1Rs differ in their level of constitutive NF-κB activation. The constitutive activity of H_1R F435L was similar to that of the WT H_1R, whereas it is hardly detectable for H_1R F435A and increased for H_1R F435V. Since mutant receptors F435L and F435V displayed basal NF-κB activation, they are not "solely activated by synthetic ligands," and therefore, only H_1R F435A fits the criteria of RASSL.

It is important to notice that ligand-independent constitutive activity of RASSLS can be apparent *in vivo*. Chronic overexpression of Ro1, a $G\alpha_i$-coupled RASSL, in transgenic mice caused mortality, independent of stimulation with the synthetic agonist (2). This mortality could be blocked by treatment with either the specific G_i-blocker pertussis toxin

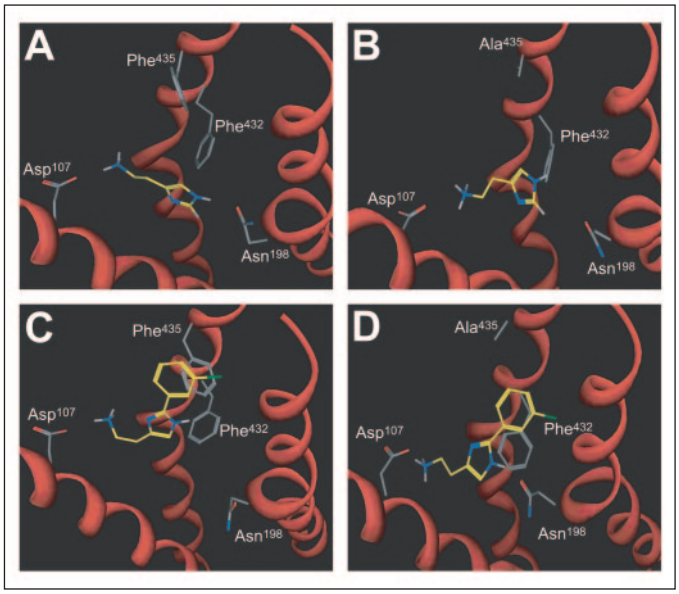


FIGURE 4. **Representative snapshots of a 1000-ps molecular dynamics run of the ligand-binding pocket of WT (*A* and *C*) or F435A H_1Rs (*B* and *D*) with either histamine (*A* and *B*) or 2-(3-chlorophenyl)histamine (*C* and *D*).**

or the Ro1 antagonist nor-binaltorphimine (2). Apparently, constitutive Ro1-signaling was accountable for this mortality, and this receptor was not a real RASSL. For the F435A-RASSL, we believe that no constitutive activity-related side effects can be expected since, even at receptor expression levels exceeding 10 pmol/mg, no basal NF-κB signaling is observed using a sensitive reporter gene assay. Besides our $G\alpha_{q/11}$-coupled RASSL (H_1R F435A), H_1R F435L may also be useful for future *in vivo* use. When replacing the endogenous H_1R, it would create a knock-out system, displaying the H_1R-like constitutive signaling but lacking histamine-induced responses. One would, however, still be able to induce H_1R responses by administration of synthetic agonists.

The *in vivo* use of the RASSL Ro1 has generated considerable knowledge on the effect of $G\alpha_i$ signaling on heart rate (1) and cardiomyopathy (2). Currently, the Ro1 has also been expressed in other tissues of transgenic mice, including several brain areas, to further address the effects of G_i signaling (6). At this moment, much less is known about *in vivo* effects of $G\alpha_{q/11}$ stimulation. Our current $G\alpha_{q/11}$-coupled RASSL may be employed to fill that gap of *in* vivo knowledge. Although the H_1R RASSL can still be stimulated by the endogenous ligand histamine at high concentrations, plasma levels of histamine in healthy humans, rats, or rabbits are well below that level (2–250 nM) (32–35). When *in vivo* application is limited to tissues in which histamine concentrations are not exceptionally high, potentially excluding, for example, brain (synaptic histamine) and immune system (mast cell contents), the H_1R RASSL is expected to remain quiescent until stimulation with synthetic agonists. Future research should focus on further modifications of our RASSL to make it even less responsive to histamine.

In conclusion, we exploited subtle differences in the binding pockets for different classes of H_1R agonists and showed that the precise orientation of histamine and 2-phenylhistamine in the binding pocket is quite different. As a result, we have thus identified H_1R F435A (6.55) as the first $G\alpha_{q/11}$-coupled RASSL. This mutant H_1R combines strongly decreased affinities and potencies for the endogenous ligand histamine with improved affinities and potencies for PheHAs, a synthetic class of H_1R agonists. In contrast to mutant H_1Rs F435V and F435L, this receptor lacks constitutive activity, which is a characteristic, we believe, that should be emphasized more strongly in the development of future RASSLs.

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